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Fate and activity of fungal BCAs delivered to strawberry flowers and their potential for integration with fungicides

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Introduction.

Grey mold caused by *Botrytis cinerea* is a serious strawberry disease. Yield loss is prevented by repeated fungicide treatments during flowering which increases the risk of pesticide residues in berries. Fruit lesions are typically initiated from *B. cinerea* infected stamens or from dead infected petals adhering to the fruit or trapped under the calyx. To implement biological control agents (BCAs) as an alternative control measure, it is crucial that the BCAs are able to colonize flower parts rapidly to combat *B. cinerea*. The combination of fungicides with BCAs may enhance and stabilise the efficacy of BCAs. The underlying mechanism for such positive combination effects may be related to an improved establishment of the BCAs when their natural competitors have been adversely affected by the fungicide or that the *B. cinerea* infection has been slowed down.

The objective of the present was to study 1) the interaction between BCAs and *B. cinerea* on strawberry flowers, 2) the sensitivity of BCAs to strawberry fungicides, and 3) the effect of combined BCA+fungicide treatment on BCAs and on the indigenous mycobiota.

Methods.

The BCAs *Clonostachys rosea* IK726, *Trichoderma harzianum* (TRI003 and Supresivit), *T. polysporum* + *T. harzianum* (Binab TF) and *Ulocladium atrum* were applied in various experiments. The interaction on flowers between *B. cinerea* and the BCAs *C. rosea* and TRI003 was examined using dual inoculations with strains of the three fungi tagged with fluorescence reporter genes encoding GFP and DsRed (Lübeck et al., 2002, Jensen et al., unpubl). Establishment of TRI003 on berries was studied in two field experiments. Flowers and green berries were sprayed with TRI003 and labelled in order to quantify establishment of *T. harzianum* on berries developing from treated flowers and berries, respectively. Fungicide sensitivity of four BCAs (*C. rosea*, Supresivit, TRI003 and Binab TF) was tested *in vitro*, using agar plates amended with six fungicides (Table 1) in six dosages (0, x1/4, x1/2, x3/4, x1 and x10) of recommended dosage. Effects on establishment of BCAs and on the indigenous microbiota by combination of BCAs (*C. rosea*, TRI003 and *U. atrum*) and the fungicide Teldor (1/2 x normal dosage) was tested in a semi-field experiment.

Table 1. Fungicides used in strawberry production in Denmark

Trade name	Active compound	Target disease	Dosage ha ⁻¹
Amistar	Azoxystrobin	Mildew	1 l
Candit	Kresoxim-methyl	Mildew	0.2 kg
Euparen Multi	Tolylfluanid	Grey mould	3 kg
Scala	Pyrimethanil	Grey mould	2 l
Signum WG	Pyraclostrobin + boscalid	Mildew+ grey mould	1.8 kg
Teldor WG 50	Fenhexamid	Grey mould	1.5 kg

Dilution plating on semi-selective media was used for quantification of fungi on flowers/berries (Jensen et al., 2013). The identity of recovered BCA strains was determined by UP-PCR fingerprinting (Lübeck et al. 2000) and ITS1-ITS2 sequencing (Sundelin et al., 2009)

Results

The ability of BCAs to germinate rapidly and in high numbers on flowers is necessary for control of grey mould. Approximately 70% of both *C. rosea* and *B. cinerea* conidia germinated on flower while only 20% of *T. harzianum* conidia germinated within 24 hours. Dual inoculation of the fungi revealed that *C. rosea* significantly reduced both *B. cinerea* and *T. harzianum* germination on flowers. In addition, *C. rosea* reduced grey mould symptoms on flowers. In two field trials labelled flowers were sprayed with TRI003. At spraying, the *Trichoderma* density varied between 10^4 - 10^5 CFU/flower, while on berries developed from labelled flowers one month later, approximately 10^2 CFU/berry was recovered. UP-PCR and ITS sequencing of DNA from pure cultures confirmed that the majority of isolates from TRI003 sprayed plots originated from the TRI003.

For successful combined application of BCAs and fungicides, the BCA strains must be insensitive to the fungicide. All the tested grey mould fungicides inhibited growth of the target pathogen *B. cinerea*, with Signum and Teldor being the most efficient fungicides inhibiting the pathogen at x1 and x1/4 of recommended dosage, respectively. Within the tested BCAs both TRI003 and Supresivit were insensitive to the all fungicides (table 1). Binab-TF was inhibited by Candit and Signum at x10 recommended dosage, but was unaffected by the other four fungicides. *C. rosea* was inhibited by Euparen and Signum at x10 recommended dosage, but was unaffected by the other four fungicides.

In a semi-field experiment the BCAs *C. rosea*, TRI003 and *U. atrum* were sprayed at the flowering stage singly or in combination with Teldor. The mycobiota on red berries examined four weeks later was dominated by *Cladosporium* spp. followed by *Penicillium* spp. and *Botrytis cinerea*. However, the densities of these fungi were not significantly affected by BCAs or by Teldor either alone or in combination with the BCAs. Furthermore, application of Teldor had no significant effect on the establishment of the BCAs. Both *C. rosea* and *T. harzianum* was recovered from berries 4 weeks after flower application but at low concentrations. The latter was also identified in plots not sprayed with TRI003. UP-PCR fingerprinting and ITS sequencing revealed that the recovered *Trichoderma* isolates from TRI003 sprayed plots originated from the product while *Trichoderma* strains isolated from other plots were indigenous. *U. atrum* was not isolated from any of the plots.

Discussion and conclusions

After a single application of *C. rosea* or TRI003 to strawberry flowers the fungi were recovered in low numbers on berries one month later. Microscopy showed that *C. rosea* germinated rapidly on flowers and restricted *B. cinerea* growth. Both *C. rosea* and TRI003 were insensitive to the fungicide Teldor. Combined BCA+Teldor treatment once at flowering did not improve BCA establishment nor did it affect the density of the indigenous fungi on berries. However, since a single application of the BCAs (\pm Teldor) resulted in a flower to berry transmission of BCAs at low densities, repeated applications e.g. weekly during flowering is expected to increase BCA establishment to a level sufficient to affect grey mold development.

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